Simultaneous Determination of Iron(III), Iron(II), and Manganese(II) in Environmental Samples by Ion Chromatography

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A chromatographic method was developed for simultaneous determination of Fe(III), Fe(II), Mn(II), and other transition metals. A high-performance polymer-coated silica-based cation exchange column was used for the separation of metals from hydrochloric acid-extracted environmental samples. After separation, the metals were mixed with PAR [4-(2-pyridylazo)resorcinol] and passed through a super-coiled Teflon postcolumn reactor. The absorbance of the colored complexes was recorded on line at 520 nm. Detection limits for Fe(III), Fe(II), and Mn(II) were 19, 9, and 25 fmol, respectively. Linear detector response was observed up to concentrations of 5—20 nmol. At concentrations of 2 nmol, the analyses were reproducible with 0.4% for Fe(III), 0.2% for Fe(II), and 0.5% for Mn(II). The method was compared with a photometric assay using ferrozine for Fe(II) determinations and hydroxylamine as reducing agent for Fe(III). Concentrations of Fe(III) were calculated from ferrozine determinations prior to and after the reduction of samples. Good agreement of both methods was obtained for various applications. Fe(III) and Fe(II) concentrations were determined in a depth profile of a flooded rice paddy soil. The profile showed increasing Fe(II) concentrations from the upper 1 mm soil layer to a depth of 10 mm, indicating the highest iron-reducing activity in a soil depth of 3 mm. A growth experiment with the metal-reducing bacterium *Geobacter metallireducens* showed Fe(III) reduction concomitant with the production of Fe(II) and consumption of acetate as a carbon and energy source. Production of Mn(II) from manganese oxide was followed with an enrichment culture of Mn(IV)-reducing bacteria.

Introduction

The valence of iron in the environment reflects its redox condition and microbial activity. The diagenesis of iron was recognized as an important biogeochemical cycle interacting with the carbon, sulfur, and nitrogen cycles. Dissimilatory iron(III) reduction is an important microbial process for organic matter degradation under anoxic conditions (*1—4*). Iron(II) oxidation on the other side occurs by chemical and photochemical reactions (*5, 6*) and is also mediated by microorganisms. Recently an anoxic Fe(II) oxidation was

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described for phototrophic (*7, 8*) and nitrate-reducing bacteria (*9*).

Natural manganese abundance is far less than that of iron. However some marine sediments are very manganese oxide-rich, and manganese reduction is an important process in these environments. Although manganese reduction in marine sediments is partly due to sulfides (*10*), the dissimilatory reduction of Mn(IV) by microorganisms was shown to play an important role in carbon oxidation in those environments (*3, 11, 12*). Manganese is typically extracted with oxalate (*13*) and analyzed by flame atomic absorption spectrometry.

The usual approach to determine Fe(II) in culture and soil experiments is to use a spectrophotometric method with ferrozine (*14*) or 2,2′-dipyridyl (*15*) as the specific Fe(II) reagent. In a second assay, the reducible Fe(III) concentration is determined by comparison of the Fe(II) concentrations prior to and after reduction of the sample. This two-stage procedure is time-consuming and labor intensive. Reduction of Fe(III) with hydroxylamine hydrochloride is incomplete in the presence of organic compounds. Luther et al. (*16*) reported that the reduction of iron(III) citrate with hydroxylamine yielded only 50—60% Fe(II) of the total iron concentration whereas the reduction of iron(III) chloride was complete. Samples containing organic matter are also problematic for the determination of oxidizable Fe(II) with redox titration using $KMnO_4$. Extracts of soil samples containing high concentrations of humics are typically dark brown, and their color might interfere with a spectrophotometric determination of Fe(II). Also chemiluminescence methods (*17, 18*) are used for specific and highly sensitive detection of Fe(II). The luminol oxidation reaction however is sensitive to Mn(II), Cu(I), and dissolved organic matter (*19, 20*). Mössbauer spectrometry is also used for the determination of the valence state of iron; however, the results obtained after a long measurement time are not quantitative.

Although ion chromatographic methods for determining Fe(II) and Fe(III) have been reported, no special emphasis was put on the determination of Fe(II) and Fe(III) concentrations of the samples at the time they were taken rather than the determination of the total iron content. Steinmann and Shotyk (*21*), using a similar chromatographic system to the one described here, observed with aging of the cation column a reduction of Fe(III) in the samples during analyses. Kanai (*22*) used a modified ion chromatographic method to analyze Fe(II) and Fe(III) in powdered rock samples. A partial oxidation of Fe(II) to Fe(III) was observed even working with anoxic samples. Oxidation of Fe(II) also seemed to occur during analyses of Fe(III) and Fe(II) by Moses et al. (*23*). They observed an artifact peak that was a function of the Fe(II) concentration of the sample and interfered with the Fe(III) peak.

Therefore, the primary focus of this work was to define conditions of reproducible sample preparation and stable elution conditions for the speciation of Fe(II) and Fe(III). The described procedure enabled the detection of iron in the original valence composition of environmental samples. The system was equipped with an autosampler that allowed automatic analyses of high numbers of samples. The method is highly reproducible and very sensitive for simultaneous Fe(III), Fe(II), and Mn(II) detection.

Materials and Methods

Chemicals. Ammonium iron(II) sulfate hexahydrate (Mohr's salt) and ammonium iron(III) sulfate dodecahydrate were used as Fe(II) and Fe(III) standards (Merck chemicals).
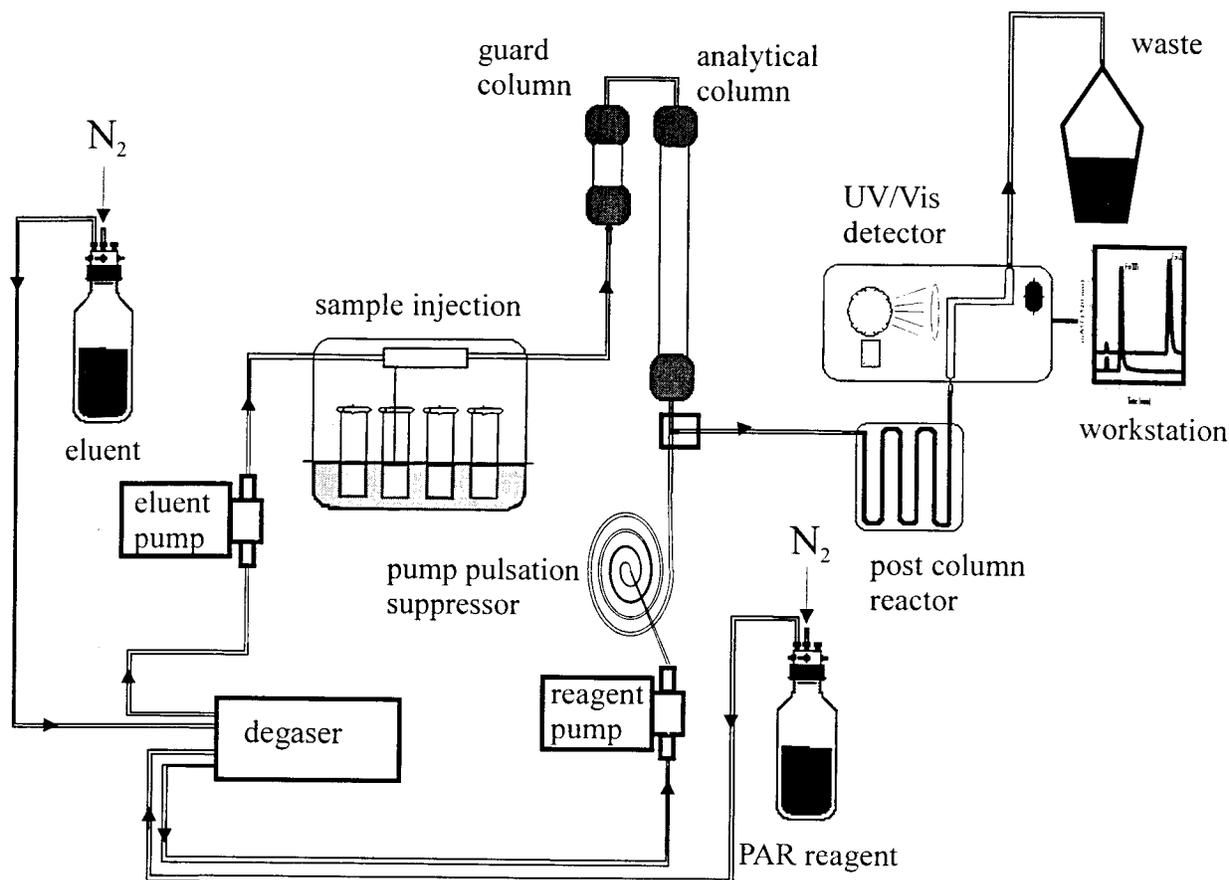
FIGURE 1. Schematic diagram of the high-performance liquid chromatographic system for metal analyses.

Manganese(II) chloride tetrahydrate from Fluka was used as Mn(II) standard. For the PAR reagent, 4-(2-pyridylazo)-resorcinol and 25% ammonium hydroxide solution (Fluka) was used. L(+)-tartaric acid (Fluka) was dissolved in ultrapure water, and the pH was adjusted to 2.7–3.1 with lithium hydroxide (Fluka) and used as eluent. For extraction, 25% hydrochloric acid (Merck) was diluted to 0.5 M. Hydrochloric acid (50 mM) was also used for dilution and as flushing reagent for the autosampler. All chemicals were analytical grade. Nitrogen (4.6) was purchased from Messer Griessheim.

Extraction of Fe(II), Fe(III), and Mn(II). Soil samples (0.5 g) were extracted with 4.5 mL of 0.5 M hydrochloric acid at room temperature for 24 h. Culture supernatant was acidified with hydrochloric acid to a final concentration of 0.5 M. During the extraction, Fe(II) precipitated in siderite, viviantite, and iron sulfide was dissolved as well as the iron oxide ferrihydrite. The iron oxides lepidocrocite and goethite were almost insoluble under these conditions depending on their crystallinity. Magnetite was not dissolved by 0.5 M hydrochloric acid. The extraction conditions were also suitable to dissolve precipitated manganese(II) carbonate from rhodochrosite but manganese(IV) oxide was not dissolved. To test the extraction procedure for iron oxidation or reduction artifacts, two different soils were used. Standard solutions of Fe(III) and Fe(II) were added at the beginning of the hydrochloric acid extraction to both rice paddy soil and forest soil with organic carbon contents of 1.8% and 9%, respectively. After the extraction (24 h), Fe(III) and Fe(II) concentrations were determined by chromatographic and photometric (ferrozine) methods and compared with the respective soils that have been extracted without iron amendments.

Ion Chromatographic System. The chromatographic system (Sykam, Gilching, Germany) as well as the sampling device were fully metal free. The sampling device consisted of two HPLC pumps (PEEK), a reagent selector with connections for nitrogen flushing, an on-line vacuum degasser, a temperature-controlled cabinet cell for the column and postcolumn reactor, and a UV–vis detector. An autosampler with a PEEK Rheodyne injection valve was used to inject 10 μL samples. The sample loop was flushed with 50 mM hydrochloric acid between injections. A PEEK HPLC column (4 × 150 mm) filled with a polymer-coated silica-based cation-exchange resin (IBJ K3 5 μm, Sykam, Gilching, Germany) was used to separate the metal ions; a guard column with neutral material (Sykam, Gilching, Germany) prolonged the lifetime of the column. Tartrate buffer (100 mM) prepared from tartaric acid, and lithium hydroxide adjusted to pH between 2.7 and 3.1 was used to elute the column at a flow rate of 1 mL min^{-1}. PAR reagent containing 1 mM 4-(2-pyridylazo)resorcinol in 4% ammonium hydroxide was mixed (0.2–0.4 mL min^{-1}) with the metals directly after they eluted from the column. For suppression of pump pulsation, a 3 m long peek capillary with 0.1 mm diameter was used to connect the pump and reactor. A supercoiled Teflon reactor (0.5 mm × 5 m) enabled effective mixing of both solutions and allowed a reaction time of 60 s before detection with a UV–vis detector at 520 nm. Eluent and PAR reagent were deoxygenated by a degasser and were both kept under nitrogen. A diagram of the chromatographic system is shown in Figure 1. The absorbance was recorded and integrated with Pyramid software (Axxiom Chromatography Inc., California). Calibrations of external standards were done using Pyramid software that also provided the calculations of concentrations from peak areas.

Photometric Detection of Fe(II) and Fe(III). The samples were extracted with hydrochloric acid as described above. For Fe(II) determination, ferrozine reagent (*14*) was used in

a slightly modified version of the technique described by Lovley and Phillips (24). Aliquots of 100 μL samples were mixed with 1 mL of ferrozine reagent consisting of 0.1% (w/w) in 200 mM N-2-hydroxyl ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer at pH 7. The mixed sample was centrifuged in 1.5 mL reaction cups at 600g for 5 min, and the absorbance of the supernatant was measured at 562 nm. As an estimation for the total iron, the extractable and reducible iron was determined by mixing 100 μL of the sample with 2 mL of a solution of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl. After an incubation of 2 h at 60 °C, an aliquot of 100 μL was mixed with 1 mL of ferrozine reagent and processed as described above. The concentration of extractable Fe(III) was determined by the difference between total extractable iron and Fe(II).

Depth Profile of Soil. Fresh Italian rice paddy soil was brought to the laboratory and was air-dried to stop microbial processes. Before the experiment, rice paddy soil was sieved (<1 mm) and mixed with deionized water. Plexiglass tubes filled with soil slurry were incubated in the greenhouse under natural illumination at temperatures between 25 and 30 °C. During this incubation, the microbial processes started again in a similar way as in the vegetation period of rice after the previous desiccation of the rice field, the rice harvest, and ploughing of the soil. After 12 weeks of incubation, profiles of Fe(III) and Fe(II) from the soil tubes were taken. Before soil layers of 1 mm thickness were cut, the cores have been placed in the anaerobic box to prevent oxidation of Fe(II) during sample preparation. All soil layers were extracted separately after the wet weight of the soil slices was determined. Parallel porewater was obtained by centrifugation of the soil slices under a nitrogen atmosphere. The supernatant was placed into HPLC vials and acidified with HCl (0.5 M end concentration) to stabilize the Fe(II) during the analysis. Porosity and density profiles of the soil were determined in triplicates with parallel soil cores.

Growth Experiment with Iron- and Manganese-Reducing Bacteria. *Geobacter metallireducens* strain GS15 obtained from the German culture collection (DSM) was grown in mineral medium containing 30 mM iron(III) citrate and 3 mM acetate. For the growth experiment, 24 bottles (50 mL) containing medium were inoculated equally and incubated at 30 °C. Three culture bottles were used for each time point. Samples of 2 mL aliquots were removed for the determination of acetate. Growth in the culture bottles was stopped by the addition of hydrochloric acid to a final concentration of 0.5 M, and Fe(III) and Fe(II) were determined after 24 h of extraction as described above.

An enrichment culture was started with acetate and manganese oxide from rice paddy soil as inoculum. After manganese oxide was dissolved, the culture was transferred repeatedly into a new medium and Mn(II) production was followed. The same extraction procedure was used for Mn(II) determinations as for Fe(III) and Fe(II) described above. Acetate was quantified by ion exchange chromatography using a refraction index detector (25).

Results

Chromatographic System for Detection of Fe(II), Fe(III), and Mn(II). The separation of transition metals was performed on a polymer-coated silica-based column. This column combined the advantage of a high column capacity (as pure silica columns, e.g., Nucleosil 5 SA) with the stability in aqueous buffers (as pellicular polymer-based ion exchangers with low capacity, e.g., IonPak CS5). This high capacity column enabled the use of a highly buffered eluent (100 mM tartrate buffer) allowing injections of acidic samples (0.5 M HCl) without any effects on peak symmetry or retention times.
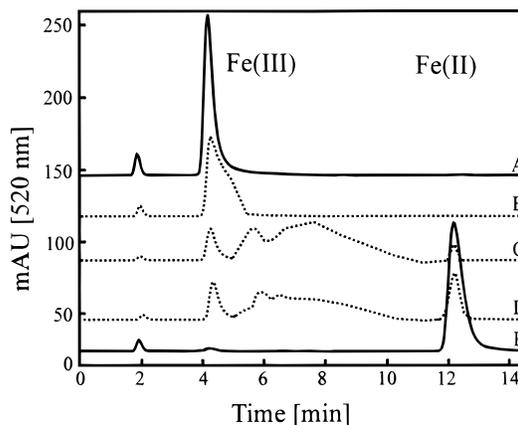

FIGURE 2. Chromatograms showing the genesis of the Fe(II) signal during the column preparation procedure with injections of 200 μM Fe(II) in 1% ascorbic acid. (A) Third injection approximately 1 h after installation and flushing of a new column. (B) Signal after 10 injections, (C) after 20 injections, (D) after 35 injections, and (E) after 50 injections. The oxidizing character of new columns could be removed by multiple injections of ascorbic acid following the Fe(II) signal during the procedure.

New columns typically showed oxidizing character and were not directly suitable for iron speciation (Figure 2). Samples of Fe(II) standards were oxidized on the column and showed signals in the front part of the chromatogram similar to Fe(III) peaks. Occasionally additional broad peaks were observed between the Fe(III) and Fe(II) peaks that were likely to be the oxidized product of Fe(II). Multi-metal standards containing Fe(III), Cu(II), Ni(II), Co(II), Fe(II), and Mn(II) showed the known elution order and retention times; however, no peak for Fe(II) was observed. This problem was solved by the following procedure to prepare new columns for analyses: Samples (50 μL) of a 1% ascorbic acid with 200 μM Fe(II) were injected about 50 times in 5−10 min intervals until a single peak was observed for Fe(II) standards. Continuous injections and elution of Fe(II) standards resulted in an initial Fe(III) peak that decreased as the Fe(II) peak increased, and finally an exclusive Fe(II) peak was observed (Figure 2). The suitability of the system was tested with injections of pure Fe(II) and Fe(III) standards to ensure that neither oxidation of Fe(II) nor reduction of Fe(III) occurred during analyses (Figure 3). After calibration with concentrations between 5 and 500 μM single Fe(III) and Fe(II) standards, the system was prepared for unknown sample analyses. Thereafter the column was routinely tested with pure Fe(III) and Fe(II) standards.

The retention times of the metals varied with the pH of the tartrate buffer. At pH 3.1, Fe(II) and Mn(II) eluted at 7 and 8.5 min, respectively. A decrease of the eluent to pH 3.0 resulted in retention times of 14 min for Fe(II) and 20 min for Mn(II). In addition to pH values, the concentration of the mobile phase also affected the retention times; with higher tartaric acid concentrations resulting in decreased retention times. Injections of pure, diluted HCl (50−500 mM) were tested for interfering signals of eluting protons causing a pH-dependent PAR absorbance at retention times close to the Fe(III) peak. HCl concentrations above 200 mM caused a small absorbance drop within the dead volume.

A typical chromatogram for Fe(III), Cu(II), Zn(II), Ni(II), Co(II), Fe(II), and Mn(II) achieved at a flow rate of 0.8 mL min^{-1} 100 mM tartrate buffer at pH 3.0 is shown in Figure 3. The peak areas were constant at different pH values of the eluent as long as the ammonia buffer concentration in the PAR reagent was high enough to provide a constant pH value in the postcolumn reactor. Changes of the eluent/PAR ratios from 4:1 to 3:2 resulted in pH variations between 9.9 and
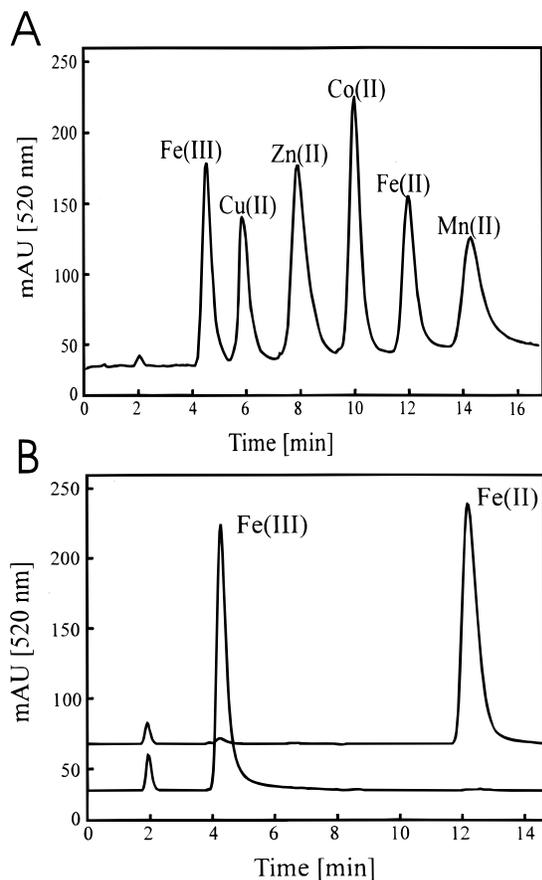
FIGURE 3. (A) Chromatogram of a six-metal standard containing 250 μM each of Fe(III), Cu(II), Zn(II), Co(II), Fe(II), and Mn(II). (B) Chromatogram of pure Fe(III) and Fe(II) standards. After the tuning procedure of new columns, no oxidation of Fe(II) was observed nor the reduction of Fe(III).

10.5. The absorbance of the Fe(III)– and Fe(II)–PAR complex was constant within that pH range, whereas the Mn(II)–PAR complex decreased below pH 10. Therefore eluent–PAR mixing ratios of 2:1 were selected for sensitive Mn(II) detection. For Fe(III) and Fe(II) detection, an eluent–PAR mixing ratio of 4:1 was most suitable because lower flow rates of PAR reagent resulted in less dilution of eluting metal peaks and therefore in a better separation and higher sensitivity.

Reaction times of 20 s for the formation of the metal–PAR complex were sufficient for maximum absorbance. With a postcolumn reactor of almost 1 mL total volume and a total flow rate of 1 mL/min, the reaction time was approximately 60 s and therefore long enough for reproducible analyses.

Reproducibility, Linearity, and Detection Limits. Iron(III) ammonium sulfate was dissolved in 0.25 M HCl and used as the Fe(III) standard. The stock solution was either diluted (5–500 μM) and analyzed directly or stored at room temperature or –20 °C for later analyses. The linear regressions did not vary with the different age and temperatures for storage (Figure 4A).

As Fe(II) standards, Mohr's salt (iron(II) ammonium sulfate) was dissolved to a concentration of 2 mM in water or in 0.25 M HCl. The stock solution was either diluted and analyzed directly or stored at room temperature or at –20 °C before dilution (5–500 μM). The calibration curves were very similar independent of the sample storage for up to 10 days at room temperature or –20 °C (Figure 4B).

Manganese(II) chloride (1 mM) was dissolved in water or in 50 mM HCl and either diluted (5–500 μM) and analyzed
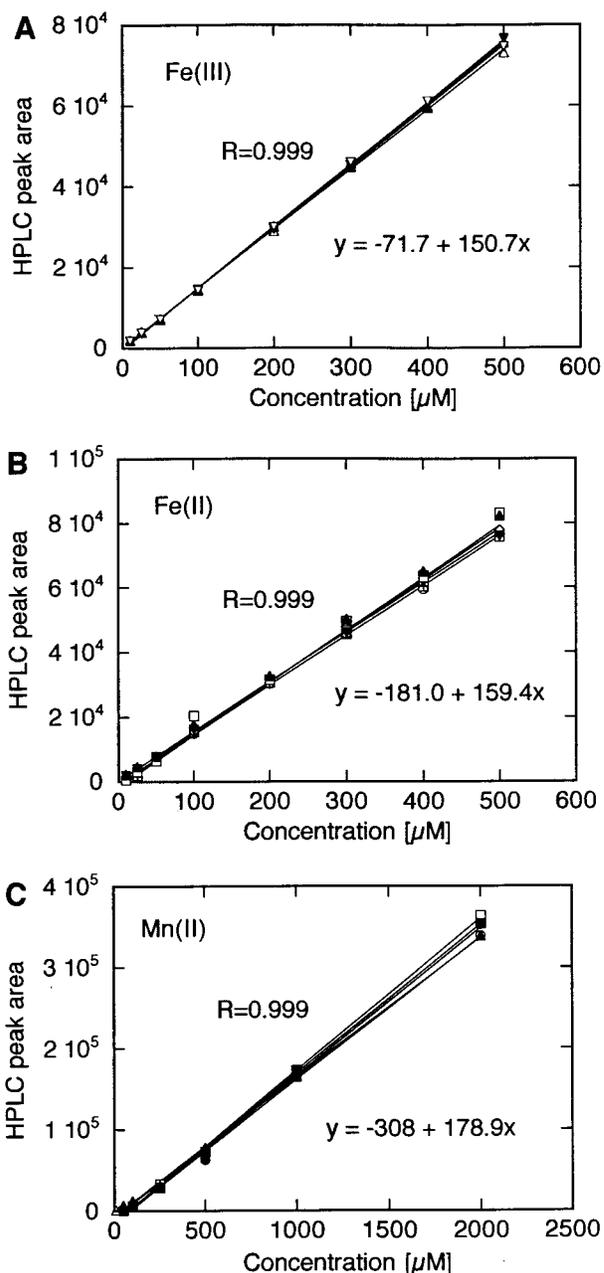

FIGURE 4. Linearity and stability of pure standards of (A) Fe(III), (B) Fe(II), and (C) Mn(II) for various experiments. (○, ●) Standards freshly dissolved and diluted in water before injection. (△, ▲) Standards freshly dissolved and diluted in 50 mM hydrochloric acid. (□, ■) Standards dissolved and diluted in water and stored at room temperature for 10 days. (▽, ▼) Standards dissolved and diluted in 50 mM hydrochloric acid and stored at room temperature for 10 days. (◇) Standards dissolved in water and stock solution stored at room temperature for 10 days and diluted before injection. (Box with plus sign inside) Standards dissolved and diluted in water, frozen at –20 °C for 10 days, and thawed before injection.

directly or stored at room temperature or –20 °C and analyzed after 2 days. The linear regression was very similar at the various conditions (Figure 4C).

The detection limit was determined by the standard deviation ($n > 10$) of the lowest calibration concentration (5 × SD). With an injection volume of 10 μL, the detection limits were 1.9 μM for Fe(III), 0.9 μM for Fe(II), and 2.5 μM for Mn(II). The detection limit for Fe(III) included the addition of 0.8 μM that was detected for Fe(III) in blank samples. No Fe(II) signals were detected in blanks. Since

TABLE 1. Analytical Results of Fe(III), Fe(II), and Mn(II) Standards from Multiple Injections ($n > 10$)

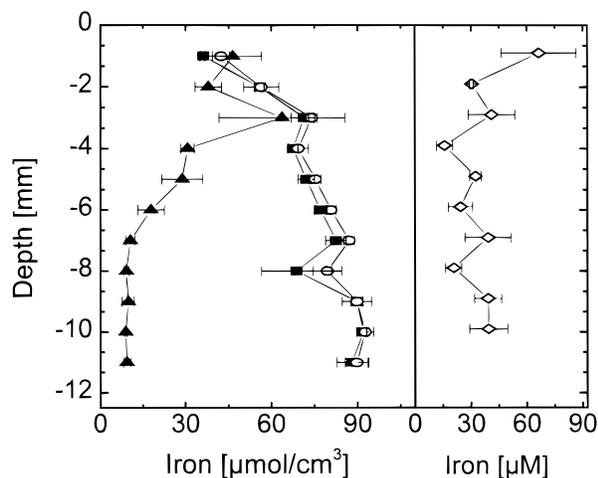| metal ion	reproducibility at various concn (μM)	relative SD (%)	detection limit (μM)
Fe(III)	500 ± 10	2	
	200 ± 0.8	0.4	
	10 ± 0.4	4	1.9
Fe(II)	500 ± 5	1	
	200 ± 0.4	0.2	
	10 ± 0.2	2	0.9
Mn(II)	500 ± 2.5	0.5	2.5

[a] Injection volume of standards 10 μL.

FIGURE 5. Depth profile of Fe(III) and Fe(II) concentrations in rice paddy soil. (Left) Fe(III) and Fe(II) concentrations after acidic extraction. Fe(II) concentrations were determined by ferrozine (■) and by ion chromatography (flattened circle). Fe(III) concentrations were determined by ion chromatography (▲). (Right) Porewater concentrations of Fe(II) determined by ion chromatography (◇). Data are means of triplicates; error bars are ±1 standard error.

the signal was linear from injection volumes from 10 to 100 μL, a decrease of the detection limit by a factor of 10 could be reached without additional precaution. The reproducibility was determined at concentrations of 10, 200, and 500 μM and varied between 0.2 and 4% relative standard deviation (Table 1).

Soil extractions with standard additions were performed to test for oxidation of Fe(II) and reduction of Fe(III) during extraction and analysis. Recovery of Fe(II) was between 90 and 110%, independent of the organic content (1.8 and 9%) of the two soils and the method of iron determination (ferrozine and chromatographic). For hydrochloric acid-extractable Fe(III), the recovery of ammonium iron(III) sulfate and ferrihydrite additions were higher than 90%.

Depth Profile of Fe(III) and Fe(II) in Waterlogged Soil. Depth profiles of Fe(III) and Fe(II) concentration in a rice paddy soil core are shown in Figure 5. Fe(II) concentrations determined after acidic extraction were lowest at the soil surface and increased continuously with increasing depth up to 10 mm. Below 10 mm depth, the Fe(II) concentrations were stable. Both iron determination methods showed identical Fe(II) depth profiles. Porewater concentrations of Fe(II) were about a factor 1000 smaller than Fe(II) concentrations after acidic extractions. Dissolved Fe(II) in the porewater showed small variations with the depth and slightly higher concentrations on the surface. Concentrations of hydrochloric acid-extractable Fe(III) decreased from the soil surface to a depth of 7 mm and remained at a concentration of 10 μmol cm^{-3} below that depth.
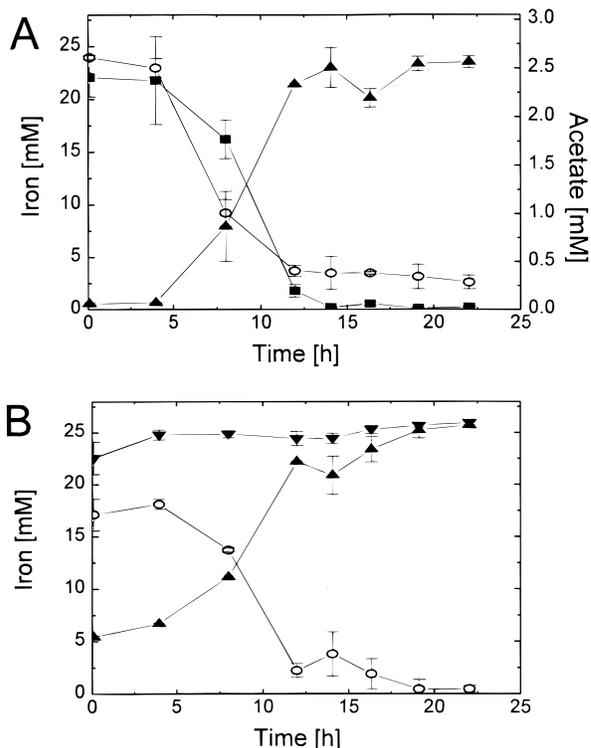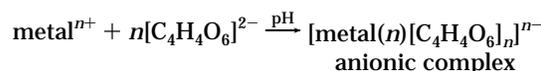
FIGURE 6. Growth experiment of the iron-reducing bacterium *Geobacter metallireducens* on acetate as electron donor. (A) Reduction of Fe(III) (○) to Fe(II) (▲) determined by the chromatographic method. Acetate (■) consumption was measured by ion exchange chromatography. (B) For comparison, Fe(II) and Fe(III) were also determined using ferrozine. Fe(II) was quantified directly (▲) and the total iron (▼) after reducing the sample with hydroxylamine hydrochloride in hydrochloric acid. Fe(III) concentrations (○) were calculated by the difference of total iron and Fe(II). Assays were performed in triplicates; error bars are ±1 standard error.

Growth Experiments with Metal-Reducing Bacteria. The reduction of Fe(III) was studied in a pure culture experiment with the iron-reducing bacterium *G. metallireducens* strain GS-15 isolated from a freshwater sediment by Lovley and Phillips (*26*). *G. metallireducens* was grown with 30 mM iron(III) citrate and 3 mM acetate in anoxic mineral medium and sampled at various times for iron determinations. The disappearance of Fe(III) paralleled acetate utilization and ceased when acetate was depleted (Figure 6A). Appearance of Fe(II) occurred concomitant with Fe(III) reduction. The results for Fe(III) and Fe(II) were similar with both methods (Figure 6B).

An enrichment culture obtained from rice paddy soil was grown with manganese oxide as the electron acceptor and with acetate as the carbon and energy source. During growth, concomitant acetate consumption and Mn(II) production were observed (Figure 7).

Discussion

The ion chromatographic method allowed an automatic and simultaneous quantification of both iron species Fe(III) and Fe(II). The elution of metals depends on the formation of anionic metal tartrate complexes from free metal cations and tartrate:

$$\text{metal}^{n+} + n[\text{C}_4\text{H}_4\text{O}_6]^{2-} \xrightarrow{\text{pH}} \underset{\text{anionic complex}}{[\text{metal}(n)[\text{C}_4\text{H}_4\text{O}_6]_n]^{n-}}$$

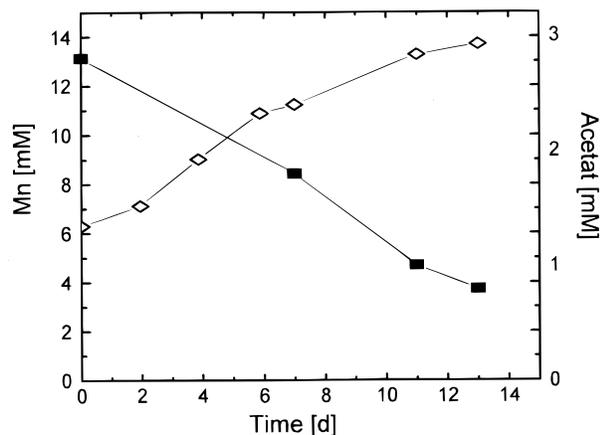The equilibrium constant of this reaction is pH-dependent.

FIGURE 7. Time course of Mn(II) production in an enrichment culture grown in anoxic mineral medium containing manganese oxide. Acetate (■) was consumed while Mn(II) (◇) was produced.

At one given pH value (e.g., pH 3.0) the equilibrium of the complex formation is much more on the complex side for Fe(III)−tartrate complex than for Fe(II)−tartrate complex (27). This is the reason for the fast elution of Fe(III) from the column since only the metal cation interacts with the cation column but not the anionic metal complex. The average abundance of free Fe(II) cations at that pH is much higher than that of free Fe(III) cations; therefore, the interaction of Fe(II) cations with the column is more frequent, and the elution of Fe(II) is much slower than that of Fe(III). This causes the atypical elution order for selected cation exchange chromatography with trivalent cations before divalent cations.

Increasing the pH value of the eluent (e.g., pH 3.1) decreases the average abundance of free metal cations and therefore decreases the retention time. The pH of the tartrate buffer is adjusted with lithium hydroxide, and the concentration of Li(I) cations in the eluent increases logarithmically with increasing pH values. At higher Li(I) cation concentrations, the interactions of metal cations with the cation column are hindered and the elution is faster. The pH-dependent equilibrium constant of the metal complex formation and the pH-dependent Li(I) concentration are both responsible for the dramatic changes in retention times with small pH shifts of tartrate buffer. Retention times of 7 and 8.5 min were observed for Fe(II) and Mn(II) with 100 mM tartrate buffer, pH 3.1, as eluent. The retention times increased to 14 and 20 min for Fe(II) and Mn(II) with tartrate buffer at pH 3.0. With tartrate buffer at pH 3.1, enough cationic character of Fe(III) to result in an elution just after the dead volume area and short retention times for Fe(II) and Mn(II) were obtained.

In the environmental samples ($n > 6000$) analyzed, 90% of all Fe(III) and Fe(II) concentrations ranged between 25 and 250 μmol (cm of soil)$^{-3}$. Extractions of these samples resulted in concentrations (50−500 μM) that were within the statistically validated calibration range. Samples from various origins were diluted when necessary. Higher injection volumes for lower detection limits (1−2 μM) were not necessary for our application. However a 10-fold increase of the detection limit could be easily achieved by increasing the injection volume from 10 to 100 μL, which showed no effect on the peak symmetry.

Oxidation/reduction of Fe(II)/Fe(III) during the analyses was recognized as a potential problem and was prevented by the acidic pH of the samples, eluent, and flushing solution of the autosampler. In addition, anoxic conditions of the eluent and PAR reagent were obtained using a degasser and nitrogen headspace of the solvents. The oxidation power of

new columns on the surface of the separation material (approximately 600 m² column^{-1}) was probably due to the production procedure using tetrahydrofuran that forms peroxides. Injections of ascorbic acid and flushing of the column with anoxic buffer established conditions for the detection of pure Fe(II) and Fe(III) standards. Re-installed columns also showed a slight oxidation of Fe(II) that could be eliminated by flushing with anoxic eluent for a few hours. Oxidation of Fe(II) was the main problem of various reports for ion chromatographic detection of iron. Moses et al. (23) describe an artifact peak in the chromatogram that only occurred if the sample contained Fe(II). This artifact peak interfered with the Fe(III) peak and made analyses of small Fe(III) concentrations impossible. Our observation with new columns showing oxidizing character is in agreement with Moses et al. (23). We also detected Fe(III) and an additional broad peak between Fe(III) and Fe(II) in samples containing exclusively Fe(II) analyzed on a new column. The broad peak was the product of Fe(II) oxidation on the column during elution. Oxidation of Fe(II) during elution was also recognized by others (27, 28). In these studies, the detection of total dissolved iron was of interest and not the speciation of iron. Therefore, the authors added ascorbic acid to the samples to reduce Fe(III) and to establish more stable elution conditions.

Sample preparation was critical to ensure the detection of Fe(III) and Fe(II) in the original ratio of the environmental sample. In rainwater analyses for transition metals, the iron was partly lost by sample filtration (29). Due to the solubility product of iron oxides, any particulate iron is often considered as Fe(III) and dissolved iron as Fe(II). However in the presence of organic compounds, Fe(III) can occur dissolved (30). Conversely, Fe(II) can precipitate in the presence of carbonate, phosphate, or reduced sulfur species. Sample filtration or centrifugation as performed for porewater analyses might yield incorrect data for both Fe(III) and Fe(II). Porewater Fe(II) concentrations are typically orders of magnitudes lower than those after extraction (Figure 5), and neither indicate iron-reducing nor iron-oxidizing activity. Therefore we chose acidic sample extraction of solid phase for Fe(III), Fe(II), and Mn(II) analyses similar to reports using various other methods [e.g., spectrophotometric detection, polarographic detection, atomic absorption, Mössbauer spectroscopy (31, 32)]. Due to the sensitivity of most cation exchange columns, acidic sample extraction has been avoided for chromatographic metal determinations in the past. The polymer-coated silica-based cation exchange column used here enabled hydrochloric acid extraction for effective solubilization of particulate Fe(II) as siderite, viviantite, and sulfide. In addition, some iron oxides were also dissolved under these conditions, allowing an estimate of the bacterial reducible Fe(III) pool of the sample. The sample dilution with hydrochloric acid stabilized dissolved Fe(II) and prevented the precipitation of Fe(III) in the sample before injection.

We routinely checked the extraction and analyses method with single species standards to ensure that neither oxidation of Fe(II) nor reduction of Fe(III) occurred during the procedure. Standard additions to soils of various organic contents at the extraction start showed Fe(III) recoveries higher than 90%. The content of humics had no effect on recovery of Fe(III) and Fe(II) although a reduction of Fe(III) has been reported in the presence of humics under acidic conditions (21, 33). With aging of the column, we observed a capacity loss resulting in a decrease of metal separations but never a reducing character of the column as Steinmann and Shotyk (21) described. They observed a broad peak as a possible reduction product of Fe(III) due to the accumulation of humics on the aging column (21).

Initially, we observed metal contamination during analyses using a chromatographic system that was fully inert except for the injection needle of the autosampler. Blank samples frequently showed Fe(III), Zn(II), Ni(II), and Fe(II) peaks in concentrations ranging up to 100 μM. The metal contamination increased with higher HCl concentrations (50−500 mM) of the sample. Substitution of the stainless steel needle by a PEEK needle drastically reduced the contamination with metals. Even blanks of high HCl concentration did not contain any metal peaks, although the injection peak increased due to the proton effect on the eluent−PAR mixture. In environmental samples, Zn(II) and Ni(II) were occasionally detected. These metals were not pursued or quantified. A mixture of six metals, however, was used routinely to check for the separation capacity of the column.

The chromatographic analysis of Fe(III), Fe(II), and Mn(II) was highly reproducible and linear over a wide concentration range (Figure 4). With automatic sample injections, the described method can become a powerful tool for iron speciation and Mn(II) detection in environmental samples. For comparison, most spectrophotometric methods (17, 34, 35) are specific for Fe(II). Fe(III) concentrations are typically calculated from total iron concentrations determined after a reduction step, and the Fe(II) concentrations are determined directly after the extraction. As reducing agents, compounds such as ascorbic acid, hydroxylamin, or sulfite are used. Similar to spectrophotometric methods, chemiluminescence methods (17, 18) are also specific for Fe(II). Spectrophotometric and chemiluminescence methods both show very low detection limits for Fe(II) in the range of 0.01−1 nM. A possible disadvantage of those methods however is the interference with other cations [Zn(II), Cu(II), Cu(I), Ni(II), and Mn(II)] and organic compounds (19, 20, 36). Matrix interferences of environmental samples (e.g., humics or other metal cations) during the chromatographic analysis described here were excluded by the combination of separation and subsequent detection of the metals. Due to the high abundance of iron in soil and sediment samples, the detection limits of this chromatographic method were at least 50−500 times lower than the typical iron concentrations in the environmental samples after extraction ($n > 6000$).

The application of the chromatographic method was tested on various soil and culture samples. The depth profile of Fe(III) and Fe(II) concentrations in flooded rice paddy soil indicated that the iron turnover in the upper 5 mm of the soil surface is highly dynamic. The Fe(II) concentration was determined independently using both the ferrozine assay and the chromatographic method, yielding almost identical absolute concentrations and depth profiles (Figure 5). Increasing Fe(II) concentrations from the soil surface to a depth of 10 mm is the result of active Fe(III) reduction in this soil zone. Only a small part of Fe(II) was dissolved in the porewater, most of it was present as $FeCO_3$. The acidic extraction dissolved solid phase Fe(II) not only from siderite but also from iron sulfide (FeS) and vivianite [$Fe_3(PO)_2$]. However due to the low sulfur and phosphorus content of the rice paddy soil, most of the solid phase Fe(II) was present as carbonate. The Fe(III) depth profile followed a reverse curve shape with highest concentrations close to the soil surface and a decrease of Fe(III) to a depth of 7 mm. Below a depth of 10 mm, both Fe(III) and Fe(II) concentrations remained constant possibly because the organic matter supporting iron reduction became limiting. The highest rates of organic decomposition are typically in the upper zone of sediments. Microbial iron reduction, however, could also be limited by easily reducible iron oxides such as ferrihydrite in depths below 10 mm. Ferrihydrite is thought to be the preferred electron acceptor for iron-reducing bacteria (32). It is the poorly crystalline iron(III) oxide typically formed by rapid oxidation of Fe(II) through chemical and microbial mediated processes (7, 9). The depth profile of Fe(III) and Fe(II) in rice paddy soil adds to the small database that supports the general importance of iron reduction in sedimentary carbon oxidation (4, 37−39).

The growth experiment with the iron-reducing bacterium *G. metallireducens* shows a second application of the described chromatographic method (Figure 6). Reduction of Fe(III) to Fe(II) with concomitant oxidation of acetate during bacterial growth confirms the experiment of Lovley and Phillips (26), who isolated and described *G. metallireducens*. The authors determined Fe(III) and Fe(II) with a photometric analyses (ferrozine) and determined acetate by gas chromatography. Independent of the methods used, both experiments showed similar results.

The chromatographic method also allowed a simple Mn(II) determination. Mn(II) production was followed with an enrichment culture growing on acetate as the sole electron donor and manganese oxide as the sole electron acceptor (Figure 7). Within 13 days, about 7.4 mM Mn(II) was produced while 2 mM acetate was consumed. This is in agreement with a complete oxidation of acetate to carbon dioxide and the reduction of stoichiometric amounts of Mn(IV) to Mn(II). Since the culture was growing in anoxic mineral medium that did not contain compounds able to chemically reduce manganese oxide [e.g., sulfide, ferrous iron (40), reduced organics (41)], we assume that the bacteria dissimilatory reduce Mn(IV) for energy conservation (42).

The described chromatographic method combined several advantages over traditional methods used for Fe(III), Fe (II), and Mn(II) determinations. Those are the simultaneous detection of the metals with high reproducibility and low detection limits. The method also allows an automatization of the analyses and excludes matrix interference in environmental samples.

Acknowledgments

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